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# The effect of the ratio of the medium volume and the number of organisms on the results of toxicological experiments

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**Abstract.** The dependence of the sensitivity of test organisms of different systematic groups and trophic levels (*Escherichia coli* bacteria, *Chlorella vulgaris* microalgae, *Daphnia magna* crustaceans, *Lemna minor* aquatic plants) to copper ions on the ratio of the volume of the medium and the number of test objects was studied. A significant decrease in EC<sub>50</sub> indices was revealed with an increase in the volume of the test sample per one organism. The possibility of increasing the sensitivity of rapid bioassays on these organisms using the equipment and methodology developed in the Siberian Federal University is shown.

## 1. Introduction

The most important characteristics of bioassays, along with their sensitivity to toxicants, are reliability and reproducibility of results [1]. However, since the late 80s of the last century, the problem of a significant variation in the results of toxicological experiments and their dependence on the conditions of conducting was discussed [2]. This question remains relevant now [1, 3]. Increasing the reproducibility of bioassay results is possible with increasing efficiency and automation of such test systems. In this regard, the Siberian Federal University is developing and improving the equipment and bioassay methodology.

In addition to the widely discussed influence on the results of bioassay physico-chemical parameters of the experiment, an important factor is the number of organisms placed in the test sample [3-8]. Using different numbers of organisms for testing samples with the same concentration of toxic substances can lead to significantly different results, since the dose of toxicant per one organism (or a unit of biomass) will be different. Thus, for microalgae, an inverse relationship was shown between the initial number of cells at the beginning of the bioassay and the toxicity of some heavy metals [5, 6].

Thus, a decrease in the number of test organisms in an unchanged sample volume (or an increase in the volume of a sample with a constant number of test organisms) may allow a substantial increase in their sensitivity to toxicants. On the other hand, the number of test organisms must ensure the possibility of reliable registration of changes in their numbers (with respect to microorganisms) and a stable reproduction of the response to toxic effects. In this regard, this article discusses the possibility of increasing the sensitivity of test organisms of different systematic groups and trophic levels (bacteria, microalgae, crustaceans, higher aquatic plants) to the model toxicant (copper ions) in order to create new rapid bioassays.



## 2. Materials and Methods

The evaluation of the toxicity of  $\text{Cu}^{2+}$  ions introduced into the samples in the form of copper sulfate was carried out on 4 test organisms: *Chlorella vulgaris* Beijer microalgae, *Daphnia magna* Straus crustaceans, *Lemna minor* L. aquatic plant and the genetically modified *Escherichia coli* bacterium strain. The response of test objects to toxic effects was assessed by the change in the following test endpoints: culture growth (*C. vulgaris*), survival rate (*D. magna*), delayed fluorescence (*L. minor*), and bioluminescence (*E. coli*). The values of all test endpoints in the tested variants containing a toxicant were expressed as a percentage of the corresponding control.

The 1-day old culture of *Chlorella vulgaris*, which is in the exponential phase of growth, was diluted with 50% Tamiya medium to an optical density of 0.063, 0.125 and 0.250, after which equal amount of inoculum was added to the control (distilled water) and test (copper sulfate solutions) variants. As a result of dilution, the concentration of the nutrient medium in all variants was 2%, and the optical density of the algae suspension was 0.0025, 0.005, and 0.01 ( $2 \times 10^4$ ,  $4 \times 10^4$ , and  $8 \times 10^4$  cells/ml, respectively). The toxic effects of copper ions were investigated in the range of 0.01-0.04 mg/l. The prepared variants were transferred in a volume of 6 ml to clean glass vials in 4 replicates, after which they were placed in multi-cell cultivators KVM-05 for cultivation under standard controlled conditions for 22 hours. Control of the growth of culture was carried out by measuring its optical density on the device IPS-03 [9].

Individuals of *Daphnia magna* of juvenile age (12-24 hours) from synchronized culture were placed in vials with 50 ml of cultivation water of 2, 6, and 18 pieces each. The number of replications for each variant of the experiment was chosen so that the total number of individuals in all test tubes was the same (54 daphnia). All test tubes were placed in a device for exposing the crustaceans (UER-03), which, thanks to the rotation of the cartridge with the flasks, ensures active gas exchange with the air medium and maintaining the same conditions for all analysed samples [10]. The device was placed in a climatic chamber with constant temperature control ( $20 \pm 1^\circ \text{C}$ ), illumination (1000-1500 lux) and photoperiod (12:12 h light:dark cycle). The toxic effects of copper ions were investigated in the range of 0.017-0.15 mg/l. Registration of the survival of crustaceans was made after 48 hours of exposure.

*Lemna minor* was maintained in a 100% (batch culture) or 2% (when conducting experiments) Steinberg nutrient medium [11]. The experiments were carried out in a climatic chamber with continuous illumination of 3000-4000 lux and a temperature of  $27 \pm 1^\circ \text{C}$ . The plants were placed in flasks containing 0.01–1 mg/l of copper ions in 4 replications so that the volume of the medium per 1 frond with 3 leaves was 4, 20, and 100 ml. The response of the photosynthetic activity of plants to the presence in the environment of a model toxicant was estimated by measuring the relative index of delayed fluorescence (RIDF) on a Foton 10 fluorometer [12] after a 20-hour exposure.

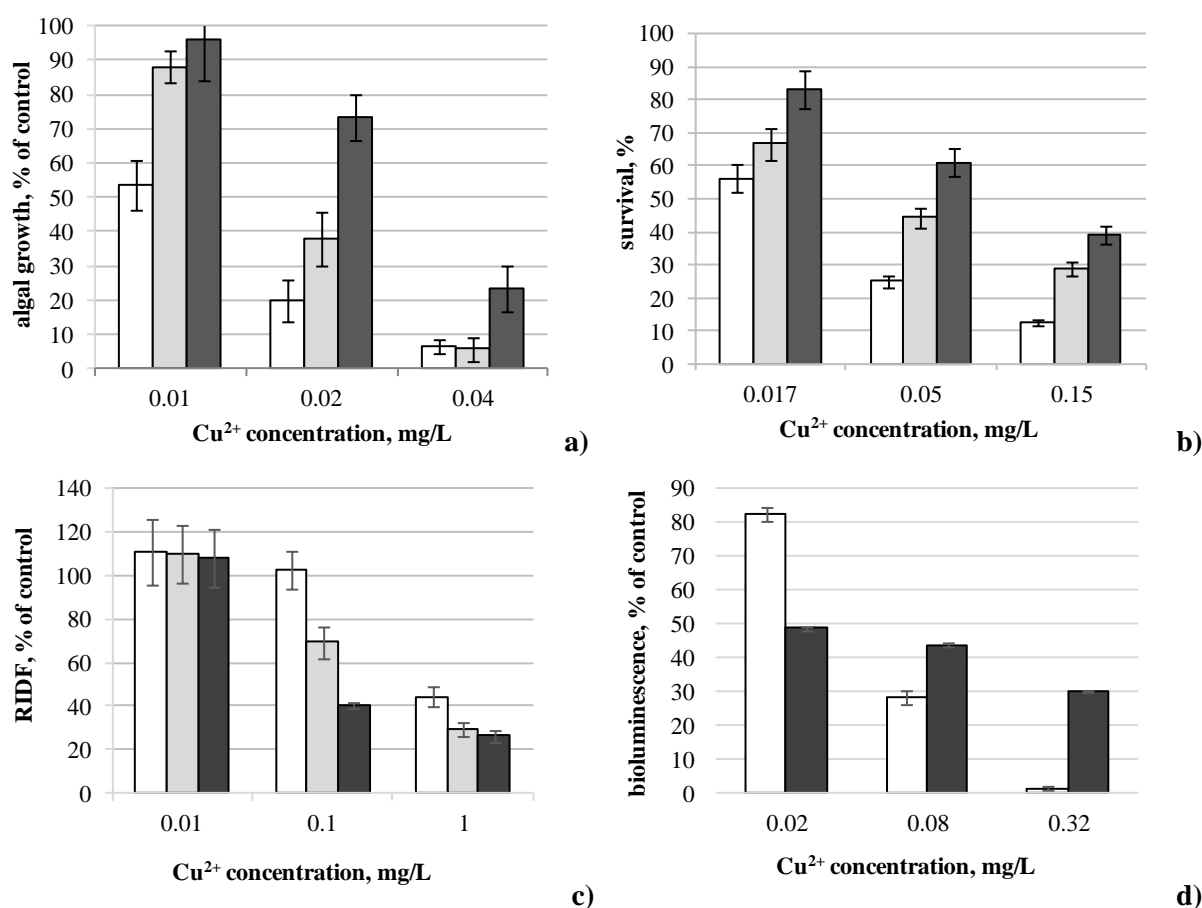
To assess changes in the level of bioluminescence of *E. coli* when exposed to copper ions, the freeze-dried bacteria Ecolum (CJSC NVO IMMUNOTECH) was used. Lyophilized bacteria were re-suspended with distilled water at the rate of 10 ml per vial of the preparation. In toxicological experiments, 0.1 ml of the obtained suspension was added to 0.9 or 6.9 ml of control and test (containing 0.02-0.32 mg/l  $\text{Cu}^{2+}$ ) variants of the experiment. The luminescence level of the bacterial suspension, reflecting their physiological state during toxic exposure, was recorded on a Foton 10 fluorimeter [12].

## 3. Results and discussion

The increase in optical density of *Chlorella vulgaris* in controls after 22 hours of intensive cultivation in KVM-05 was  $51 \pm 3$ ,  $49 \pm 4$  and  $36 \pm 4$  times with the initial cell number in one ml of medium  $2 \times 10^4$ ,  $4 \times 10^4$  and  $8 \times 10^4$ , respectively. The data obtained indicate that an increase in initial cell density caused a slowdown in the growth of the test culture, probably due to the depletion of  $\text{CO}_2$  and nutrients in the medium by the end of the bioassay. The results of the experiments (figure 1a) also indicate an increase in the toxic effect of copper ions with a decrease in the initial number of cells.

The survival rate of crustaceans in all control variants by the end of exposure remained at the level of 100%. This result confirms that, under rotational conditions, an increase in the density of organisms

from 2 to 18 does not cause significant stress, which in stationary conditions may be caused by the deficiency of oxygen dissolved in water. At the same time, the results of experiments showed (figure 1b) that an increase in the number of test organisms per unit volume led to an increase in the survival of crustaceans in the presence of a model toxicant. Similar values of survival were observed in variants of the experiment, in which a 3-fold increase in the concentration of copper ions corresponded to a 3-fold increase in the density of planting.



**Figure 1.** Effect of copper ions exposure to the test endpoints: a) *C. vulgaris* algae growth (initial density of cells in 1 ml □ 2 × 10<sup>4</sup>, ▒ 4 × 10<sup>4</sup>, ■ 8 × 10<sup>4</sup>); b) *D. magna* survival rate (the number of crustaceans in a unit of volume □ 2 un., ▒ 6 un., ■ 18 un.); c) RIDF level of *L. minor* (medium volume □ 4 ml, ▒ 20 ml, ■ 100 ml); d) bioluminescence level of *E. coli* (medium volume □ 0,9 ml, ■ 6,9 ml).

When copper ions were introduced at a concentration of 0.1 mg/l in a medium with a volume of 4 ml, there was no significant difference in the RIDF of the duckweed from the control variant (figure 1c). At the same time, with an increase in the volume of the medium in which the test organisms were located, an increase in the toxic effect was observed. In particular, the same concentration of Cu<sup>2+</sup> caused a decrease in the RIDF level by 30% and 60% in media of 20 and 100 ml, respectively (figure 1c).

The results of experiments on the effect of copper ions on the level of bioluminescence of *E. coli* bacteria in different volumes of the medium showed a common trend for all test organisms (figure 1d). The maximum concentration of copper ions (0.32 mg/l) suppressed the level of luminescence of bacteria to the factor of 3 and 100 when the media volume was the 10 and 70 times greater than the inoculums volume, respectively.

**Table 1.** EC<sub>50</sub> values of copper ions for 4 test organisms with varying ratio of medium volume and number of organisms.

C. vulgaris	relative volume of medium, µl/cell	0.300	0.150	0.075
	EC <sub>50</sub>	0.011	0.017	0.028
D. magna	relative volume of medium me, ml/crustacean	25.00	8.33	2.78
	EC <sub>50</sub>	0.02	0.04	0.09
L. minor	relative volume of medium, ml/frond	100	20	4
	EC <sub>50</sub>	0.07	0.31	0.78
E. coli	relative volume of medium, ml/0.1 ml of bacterial suspensions	6.9	0.9	–
	EC <sub>50</sub>	0.019	0.06	–

The EC<sub>50</sub> values of copper ions obtained from graphical data for 4 test organisms (figure 1) and the corresponding ratios of volumes of media and organisms are presented in table 1. For convenience of comparison, these volumes of media are listed to 1 test organism (alga cell, crustacean or a duckweed frond) or the same inoculum volume (bacteria suspension). The data presented in the table indicate a significant increase in the EC<sub>50</sub> indices with a decrease in the volume of the medium per test-organism. The results obtained by us are consistent with the data on the increase in the EC<sub>50</sub> of copper ions with an increase in the initial cell density in longer (72 h) bioassays on microalgae [5]. The reason for reducing the impact of toxicants on test objects with an increase in their number in the sample is that in terms of its fixed volume, each organism has a smaller amount of toxic substance. In addition, as a result of the absorption of the toxicant molecules, its concentration in the sample decreases. At the same time, the greater the number or mass of test organisms used in the experiment, the stronger the decrease in the concentration of the active substance in the analysed sample. With a small amount of toxicants, their concentration in the tested sample in a toxicological experiment can be reduced to zero values. In such a situation, an increase in the duration of exposure will not allow an increase in the toxic effect and, as a consequence, the sensitivity of the bioassay conducted.

## References

- [1] Azizullah A and Häder D-P 2017 A comparison of commonly used and commercially available bioassays for aquatic ecosystems *Bioassays: Advanced Methods and Applications*
- [2] Nyholm N and Källqvist T 1989 Methods for growth inhibition toxicity tests with freshwater algae *Environmental toxicology and chemistry* **8** 689-703
- [3] Lambert A S, Pesce S, Foulquier A, Gahou J, Coquery M and Dabrin A 2015 Improved short-term toxicity test protocol to assess metal tolerance in phototrophic periphyton: toward standardization of PICT approaches *Environ Sci Pollut Res* **22** 4037–45
- [4] Moreno-Garrido I, Lubián L M and Soares A M 2000 Influence of Cellular Density on Determination of EC<sub>50</sub> in Microalgal Growth Inhibition Tests *Ecotoxicology and Environmental Safety* **47** 112-6
- [5] Franklin N M, Stauber J L, Apte S C and Lim R P 2002 Effect of initial cell density on the bioavailability and toxicity of copper in microalgal bioassays *Environmental Toxicology and Chemistry* **21**(4) 742–51
- [6] Slaveykova V I 2007 Predicting Pb bioavailability to freshwater microalgae in the presence of fulvic acid: Algal cell density as a variable *Chemosphere* **69** 1438–45
- [7] Hong Y, Hu H-Y and Li F-M 2008 Growth and physiological responses of freshwater green alga *Selenastrum capricornutum* to allelochemical ethyl 2-methyl acetoacetate (EMA) under different initial algal densities *Pesticide Biochemistry and Physiology* **90** 203–11
- [8] Araújo C V M, Pereira K C and Blasco J 2018 Avoidance Response by Shrimps to a Copper Gradient: Does High Population Density Prevent Avoidance of Contamination

- Environmental Toxicology and Chemistry* **37(12)** 3095–101
- [9] 2014 *Measurement of the optical density of a culture of alga Chlorella (Chlorella vulgaris Beijer) to determine the toxicity of drinking, fresh natural and wastewater, water extracts from the soil, soil, sewage sludge, production and consumption waste* (Moscow)
- [10] Grigoriev Yu S and Shashkova T L 2018 *Biotesting method of toxicity of water on lower crustaceans* The patent for the invention No 2377560 (Moscow: Rospatent) vol 36
- [11] ISO/DIS 20079 Water quality Determination of the toxic effect of water constituents and waste water to duckweed (*Lemna minor*) Duckweed growth inhibition test.
- [12] 2014 *Methods of measuring the relative rate of delayed fluorescence of the chlorella algae culture (Chlorella vulgaris Beijer) to determine the toxicity of drinking, fresh natural and wastewater, water extracts from soils, soil, sewage sludge, production and consumption waste* (Moscow)